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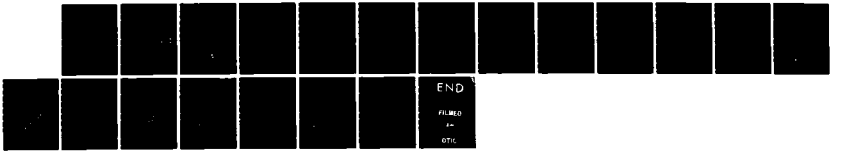

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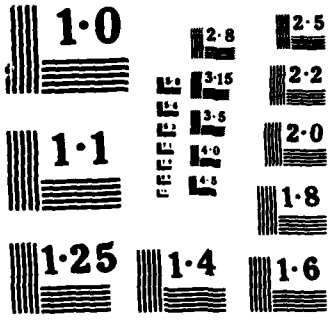

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Ion Transport Across Synthetic and Biological Membranes: Ionophore-mediated transport		5. TYPE OF REPORT & PERIOD COVERED Final Report May 1982 - September 1985
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Lea Elau		8. CONTRACT OR GRANT NUMBER(s) N00014-82- K-0437
9. PERFORMING ORGANIZATION NAME AND ADDRESS Stern College for Women Yeshiva University 245 Lexington Avenue, New York NY 10016		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS		12. REPORT DATE October 21, 1985
		13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report)
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

DISTRIBUTION STATEMENT A
Approved for public release;
Distribution Unlimited

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Presented at conferences: 8th International Biophysics Congress, Bristol U.K. 1984; 2. 189th National Meeting of the American Chemical Society, Miami Beach, Fl., 1985. Published Biochin Biophys Acta 778,219 (1984)

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Ionophores; A23187; X537A ; Lariat ethers, Arsenazo III; Phosphatidy/choline, Cholesterol, Large unilamellar vesicles, Membrane permeability, Ion transport, Ca^{2+} -transport

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Our objectives were: (1) to set-up a research laboratory; (2) to initiate membrane related research; (3) to investigate ionophore-mediated transport and (4) to involve students in research.

The laboratory has been equipped and set-up and research is performed on a regular basis with continuous student involvement.

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Block 20

Synthetic compounds, the lariat ethers and a non-cyclic di-n-propyl diamide were investigated. These compounds were known to form complexes with cations in organic solvents however, their ionophoretic activity in model membranes has not been previously demonstrated. We were able to show that some lariat ethers mediate potassium transport across phosphatidylcholine bilayers and that the diamide induced calcium movement across lipid membranes.

Potassium transport was followed using an ion sensitive electrode whereas calcium transport was measured by using the absorbance changes of arsenazo III. The feasability of fluorescence measurement using carboxyfluorescein were considered as well.

The polyether carboxylic acids A23187 and X537A were extensively investigated. The initial rates of calcium movement across lipid bilayers were measured as a function of the ionophore and calcium concentration. It has been found that the transported complex has a stoichiometry of ionophore:calcium 2:1 and 1:1 for A23187 and X537A respectively. In both systems presence of about 25 mol % of cholesterol in the bilayer reduced the initial rates. The rate reduction was 35±2% with A23187 as the ionophore and 56±1% in presence of X537A. It was also shown that A23187 is approximately 100 fold more potent as a calcium ionophore than X537A.

However, esterification of the carboxylic group of A23187 to yield the appropriate methyl ester reduced the potency more than 200 fold and the stoichiometry was changed to ionophore: calcium 1:1.

Ion Transport Across Membranes

Final Report

The objectives of this project were to set up a laboratory with the necessary equipment for membrane related studies, to initiate research in the chemistry department of Stern College for Women with active student involvement, and to conduct research on ionophore-mediated cation transport across membranes.

At present, we have a successfully operating research laboratory with sufficient equipment for performing most of the experiments. During the 1982/83 academic year Renee B. Stern, a senior in our school at that time was involved in the investigation of the A23187-mediated calcium efflux from phosphatidylcholine vesicles. She was a co-author in our 1984 article (1) and her results were included in our presentation at the International Biophysics Congress (2). At present, Ms. Stern is a Ph.D. student at the Sue Golding Graduate Division of Medical Sciences of Albert Einstein College of Medicine. In her initial interview she was asked to discuss her research and this played an important role in her acceptance to the school. Ms. Elene A. Gutman worked in our laboratory during the 1983/84 academic year and the fall semester of 1984, she graduated in June 1985. Her work dealt with the X537A-mediated transport of calcium across lipid membranes. Ms. Gutman's results were submitted for presentation at the Student Affiliates Research Symposium held at the National Meeting of the American Chemical Society (3). Her oral presentation won first prize in the Biochemical Section and was awarded third prize when all sections were considered. She started her graduate studies in the Ph.D. program of Case Western Reserve University.

These students, at the start of their research in my laboratory, did not have clear career goals. Their exposure to research, supported by your agency, was a major factor in their decision to pursue a career in science at the doctorate level.

A23187 and X537A

Calcium transport across egg phosphatidylcholine (PC) bilayers mediated by the polyether carboxylic acid ionophores A23187 and X537A was studied in large unilamellar vesicles (LUV) by following the absorbance change at 650 nm of the calcium sensitive dye arsenazo III. The concentration of arsenazo III when in the external medium was 0.18 uM. The vesicles were prepared by slow injection of an ethanolic solution of lipids into a stirred aqueous buffer solution containing 0.10 M calcium chloride or 12-20 mM arsenazo III. The external medium was replaced by isotonic choline chloride solution by passing the vesicles through a Sephadex G-50 (medium) column (1 x 40 cm). Efflux studies were conducted by adding arsenazo III to the suspension containing the calcium trapping vesicles whereas influx studies were performed by adding clacium chloride to the sample containing arsenazo III trapping vesicles.

Initial rates were claculated from the linear portion of the plots of absorbance vs time (see inset to Fig. 1 for a typical example). Fig. 1 shows the rate of calcium efflux as a function of A23187 concentration and it also shows that incorporation of 26 mol % cholesterol into egg PC vesicles causes a decrease of approximately 37 % in the rate of calcium efflux at 22° and at 0.012 uM to 0.12 uM A23187.

Using a log-log plot of the initial rate of calcium transport vs A23187 or calcium concentration (Fig. 2) we showed that the anion of A23187 forms a 2:1 ionophore:calcium complex in egg PC bilayers.

Similar experiments were conducted with a less active derivative of A23187 in which the carboxylate group was esterified (CH3A23187). This derivative forms a 1:1 complex with calcium as indicated by a log-log plot (Fig. 3) similar to the ones described above. The CH3A23187 is a much less potent calcium ionophore than A23187, at 0.096 uM the calcium efflux was about 220 fold faster in presence of A23187 than in presence of CH3A23187.

Similar studies were carried out with X537A. The rates of X537A-mediated calcium influx into the vesicles were measured as a function of calcium concentration. At 14 uM X537A the initial rates of 5.0×10^{-3}, 10×10^{-3} and 25×10^{-3} absorbance units per min were found at 10, 20 and 43 mM calcium ion concentration respectively. A log-log plot of initial rates of calcium efflux vs X537A concentration (Fig. 4) indicated a first order dependence of the X537A-mediated calcium transport across the membrane. Therefore, it was assumed that the stoichiometry of the complex transported across the bilayer under the conditions of the experiment is ionophore:calcium 1:1. Incorporation of 25 mol % cholesterol into the vesicles at constant total lipid concentration induced a decrease of about 55 % in the initial rate of calcium efflux (Table I)

TABLE I

The effect of cholesterol on the initial rates of calcium efflux from egg PC vesicles[a]

Ionophore	(uM)	Cholesterol[b]	Initial rate dA/dt(a.u. min^{-1})[c]	Reduction of rate (%)
A23187	0.048	-	1.2×10^{-2}	
	0.048	+	0.8×10^{-2}	33
	0.096	-	4.9×10^{-2}[e]	
	0.096	+	3.1×10^{-2}	37
X537A	0.096[d]	-	0.4×10^{-3}[e]	
	5.8	-	5.5×10^{-2}	
	5.8	+	2.5×10^{-2}	55
	11.6	-	10.5×10^{-2}	
	11.6	+	4.5×10^{-2}	57

a. Total lipid concentration about 0.1 mM
b. Cholesterol was present at 25±1 mol %
c. Absorbance units per minute
d. Extrapolated from results using higher concentrations of ionophore.
e. Relative initial rate of A23187/X537A122/1.

The reduction of the rates of calcuim movement across the cholesterol-containing lipid bilayers is in agreement with the effect of cholesterol on membranes, that of tightening them by increasing the packing of the component molecules. The cholesterol reduced the rates almost twice as much when the calcium transport was mediated by X537A compared to the A23187-

mediated transport. However A23187 was found to be a much more effective calcium ionophore. The initial rate of calcium efflux from egg PC vesicles was more than 100 times higher than the rate induced by X537A at 0.096 uM ionophore (Table I).

Lariat ethers

The potassium ion efflux from potassium trapping LUV, mediated by lariat ethers (supplied by Dr. George Gokel, Fig. 5) and by valinomycin was followed by using an Orion Ionalyzer model 901 equipped with a potassium electrode. Readings of potassium concentration in the external medium were recorded on a printer attached to the instrument. Initial rates were calculated from data obtained every 6 seconds after the addition of the ionophore and the results are presented in Table II.

Table II

Initial rates of potassium efflux from dimyristoyl-PC vesicles[a]

Ionophore[b]	$d[K^+]/dt$ (10^8 mole/min)	Relative rates[c]
-	<0.1	0.1
Valinomycin	0.9	100
1	0.2	0.2
2	2.3	2.3
3	0.2	0.2
4	0.1	0.1
5	0.1	0.1
6	0.4	0.4
7	1.4	1.4

a. Dimyristoyl-PC 0.75 ±0.05 mM; intravesicular potassium chloride concentration 0.15 M; Temperature 28°
b. Valinomycin 3 uM other ionophores 0.33 mM. Structures and names in Fig. 5.
c. Results normalized to 0.33 mM.

Carboxyfluorescein

Cotransport of carboxyfluorescein (CF) anion may be observed in ionophore-mediated cation transport provided that the ionophore-cation complex is positively charged. CF leakage from vesicles containing both the cation under investigation and CF may be followed by following the increase of the fluorescence as CF becomes diluted in the external medium (its fluorescence is quenched while trapped in the vesicles). Since we did not have a fluorescence spectrophotometer, preliminary data were obtained, on this project, using absorbance measurements. Aliquots of 0.3 ml of small unilamellar vesicles trapping potassium and carboxy-fluorescein were placed in dialysis bags, these bags were placed in vials containing 3.0 ml isotonic buffer solution. To the control samples 10 ul ethanol was added and to the other 1.8×10^{-3} u mole valinomycin in 10 ul ethanol was added. The vials were shaken at 25°. Aliquots were removed from the external medium for absorbance measurements per'ormed at 490 nm. Within about 80 min there was no more absorbance increase in the sample, the control reached approximately the same absorbance value within 4 hours. Absorbance measurements from 5 to 30 min after the addition of valinomycin indicated that about three times as much CF leaked from the ionophore containing vesicles than from the ethanol containing vesicles. The higher amount of CF on the outside in presence of valinomycin may be associated with the cotransport of CF along with the valinomycin-potassium complex.

Therefore this method will be considered in some future studies.

N,N,N',N',-tetrakis-(n-propyl)-3,6-di(N-methylazaoctaneacetamide)

The above named non-cyclic amide (DMED-PR) was supplied by Dr. I.J. Borowitz. Its structure is given in Fig. 5. DMED-PR was capable of binding divalent cations and extracting them from an aquoeus solution to organic solvents. Its activity as a calcium ionophore was investigated in our laboratory. Much higher concentrations (of about 10^4 fold) of DMED-PR were required to produce rates of calcium transport similar to that induced by A23187. The initial rates of DMED-PR -mediated calcium transport doubled with a two fold increase in the ionophore concentration up to about 0.5 mM DMED-PR. This experiment was carried out in small unilamellar PC vesicles. At concentrations higher than 0.5 mM the increase in the intial rate was no longer linear (Fig. 6). Presence of cholesterol reduced the rate of DMED-PR induced calcium efflux (Fig. 7). Further research in this compound is needed in order to elucidate its action as a calcium ionophore.

Ionomycin

Ionomycin is the most potent calcium ionophore known. It has become recently available from commercial sources. Due to the high activity of this ionophore our previous methods were unsuitable for initial rate measurements in egg PC vesicles. Rate reduction was achieved by cholesterol incorporation in the vesicles, reduction of the temperature and the use of extremely low concentrations of ionomycin. The results are summarized in Table III.

TABLE III

Ionomycin-mediated calcium efflux from egg PC vesicles

[Ionomycin]	Initial Rates dA/dt (a.u.min^{-1})		
uM x 10^2	Vesicle 1		Vesicle 2
	18°C	25°C	25°C
0.58	0.03	0.12	0.01
1.74	0.05	0.14	0.01
3.49	0.10	0.18	0.02
11.6		0.28	0.03
34.9	0.16	0.45	0.04

Total lipid concentration was 0.12 mM

Summary

Cation movement across lipid bilayers has been investigated. Some work has been done on synthetic compounds that induced potassium leakage in phospholipid vesicles. However, most of the research concerned calcium permeability across lipid membranes. Arsenazo III was used in all these experiments but some other methods, that eventually might be useful in these investigations were studied. Preliminary experiments were performed using DMED-PR and ionomycin. The most useful results were obtained with A23187 and X537A. In our research we were able to solve some previously unanswered problems. Most of the results were published and/or presented at scientific meetings. At present, in our laboratory there is sufficient instrumentation and other related systems for carrying out research on ionophore-mediated ion transport in phospholipid vesicles.

Abbreviations: PC, phosphatidylcholine; LUV, large unilamellar

vesicles; CF, carboxyfluorescein; DMED-PR,N,N,N',N'-tetrakis-(n-propyl)-3,6-di(N-methylazaoctaneacetamide).

References

1. L. Blau, R.B. Stern and R. Bittman (1984) Biochim. Biophys. Acta 778, 219.

2. L. Blau, R.B. Stern, T-C. Wun and R. Bittman (1984) 8th International Biophysis Congress, Bristol, U.K.

3. E.A. Gutman and L. Blau (1985) 6th Student Affiliates Research Symposium at the 189th National Meeting of the American Chemical Society, Miami Beach, Fl.

Figure Captions

Fig. 1. The initial rates of calcium release from LUV prepared from egg PC as a function of the ionophore concentration. (●), 0.063 mM PC; (▼), 0.15 mM PC; (o), 0.075 mM PC, 0.027 mM cholesterol. Inset: Absorbance changes of arsenazo III solution at 650 nm resulting from A23187-mediated calcium efflux from LUV as a function of time. The concentration of PC and A23187 were 0.076 mM and 0.06 uM respectively.

Fig. 2. Dependence of rates of calcium efflux and influx on A23187 and calcium concentrations. (A) A plot of the log of the initial rate of calcium release from LUV vs the log of A23187 concentration. Symbols and lipid concentrations are as indicated in the caption to Fig. 1. (B) A plot of log of initial rate of clacium influx into LUV as function of log of calcium concentration. The PC concentration was 0.12 mM.

Fig. 3. Effect of log CH3A23187 concentration on the log of initial rate of calcium efflux from LUV. The PC concentration was 0.060 mM.

Fig. 4. Effect of log X537A concentration on the log of initial rate of calcium efflux from LUV. The PC concentration was 0.076 mM.

Fig. 5. The names and structures of crown and lariat ethers and DMED-PR.

Fig. 6. Initial rates of calcium ion efflux from LUV as a function of DMED-PR concentration. PC 0.075 mM and cholesterol 0.027 mM.

Fig. 7. Initial rates of calcium ion efflux from small unilamellar vesicles as a function of DMED-PR concentration; (●) PC, 0.13 mM; (o) PC, 0.11 mM and cholesterol, 0.03 mM.

FIGURE 1

The Initial Rates of Ca^{2+} Efflux from Egg-PC LUV as a Function of the Ionophore Concentration

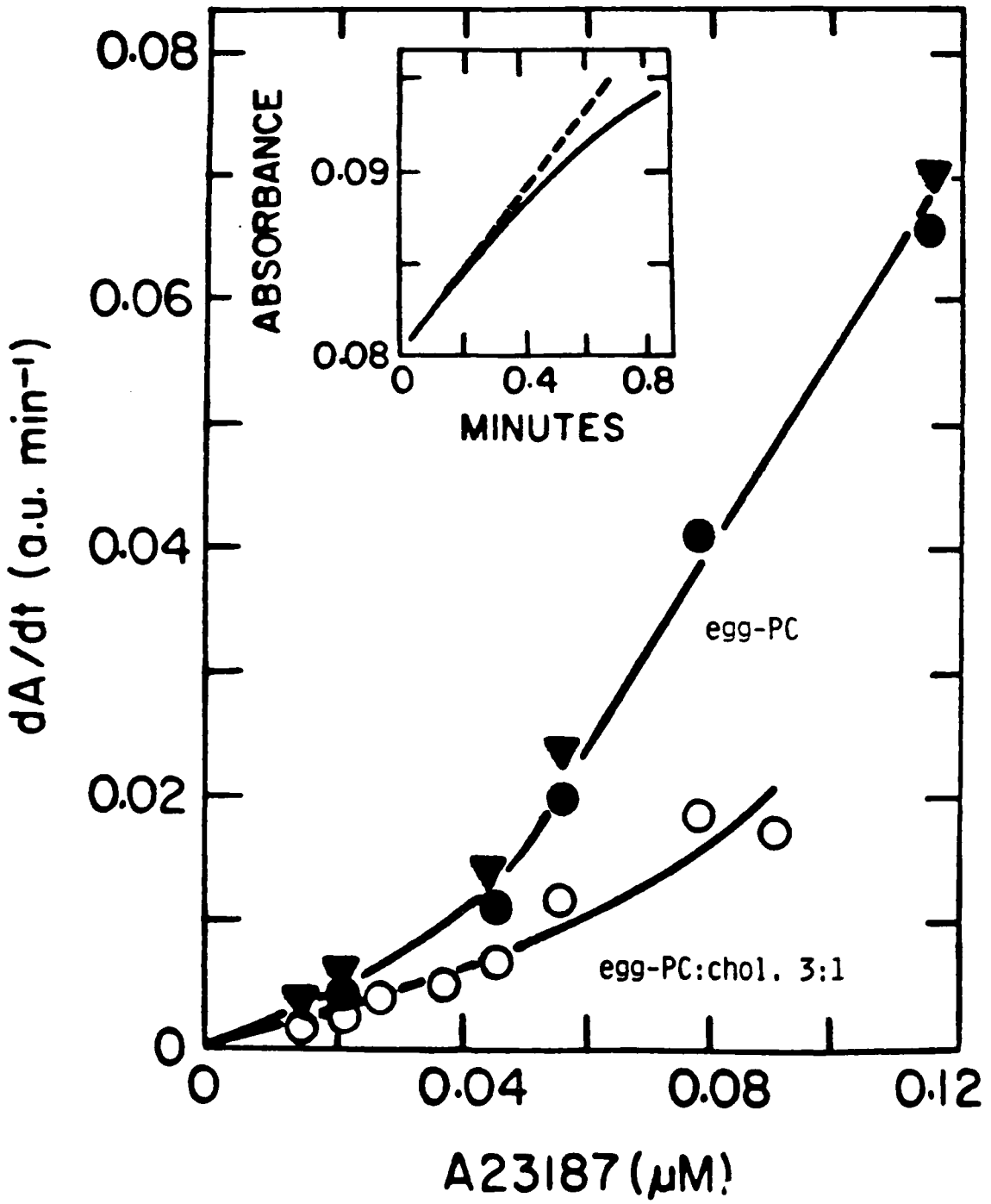
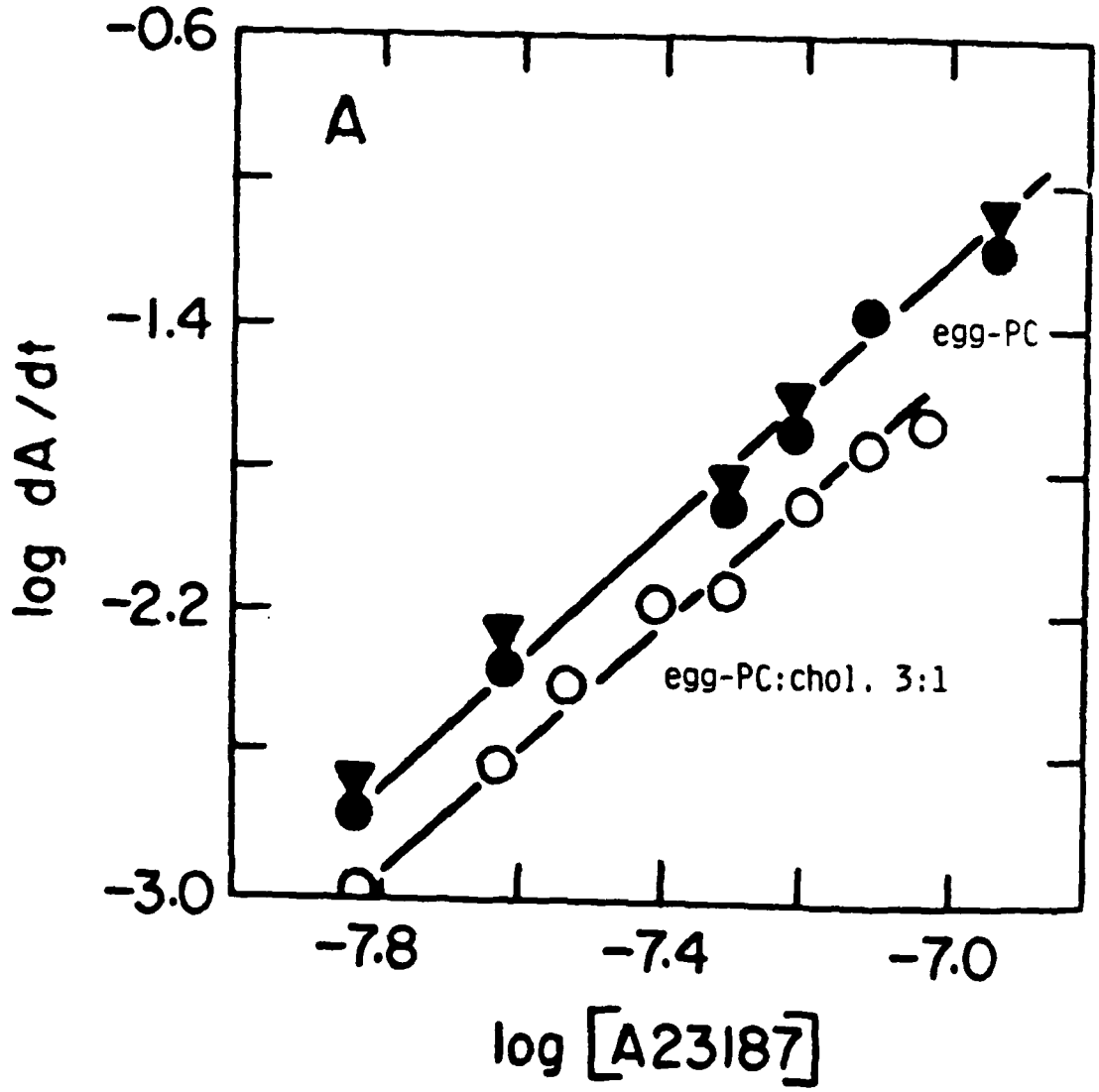


FIGURE 2

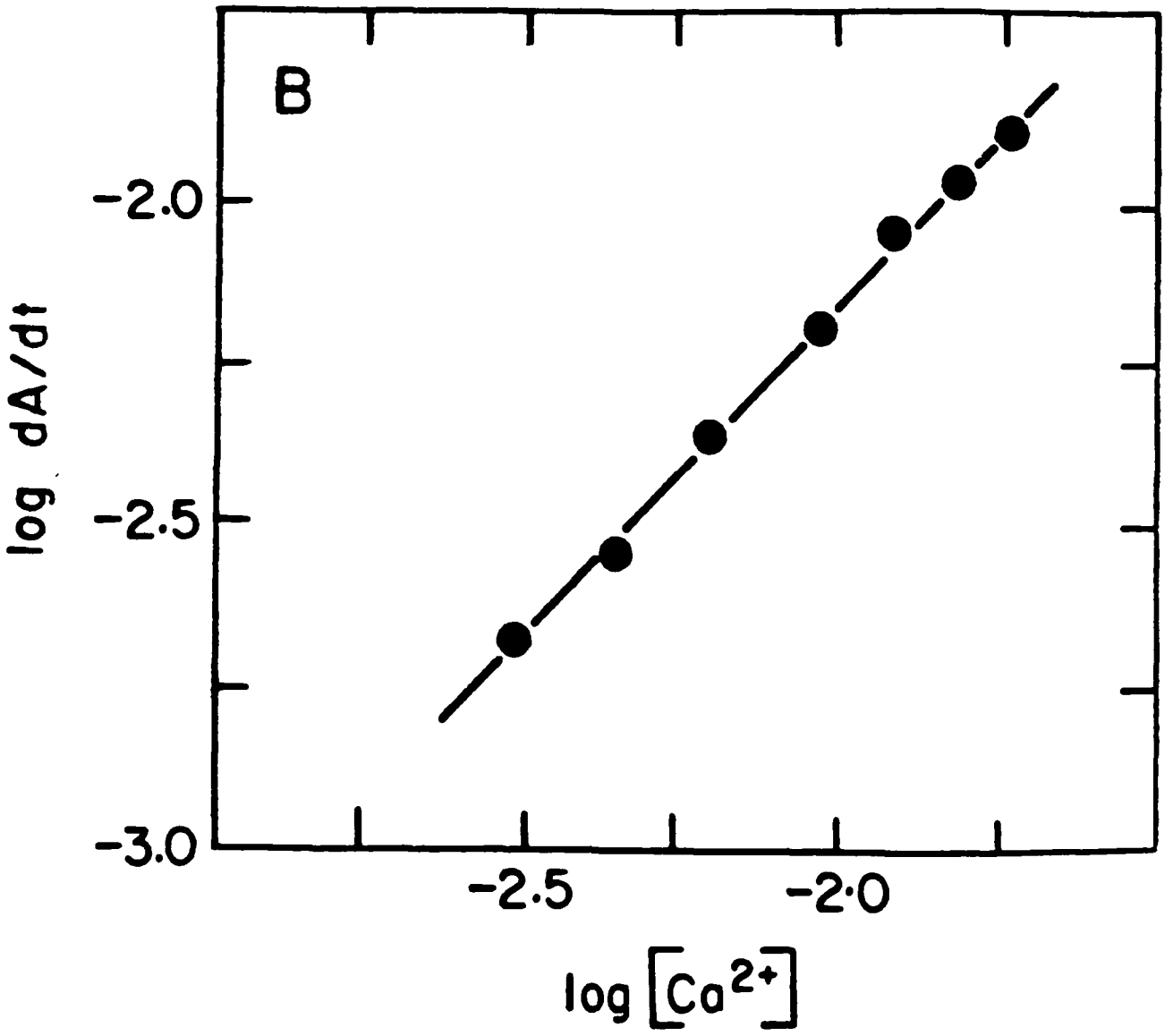
The log-log Plot of the Initial Rates of Ca^{2+} Efflux from LUV as a Function of the A23187 Concentration



Slopes 1.9±0.1

FIGURE 2

The log-log Plot of the Initial Rates of Ca^{2+} Influx into LUV as a Function of the Ca^{2+} Concentration



Slope 1.05±0.06

FIGURE 3

The log-log Plot of the Effect of the Ionophore Concentration on the Initial Rates of Ca^{2+} Efflux from LUV

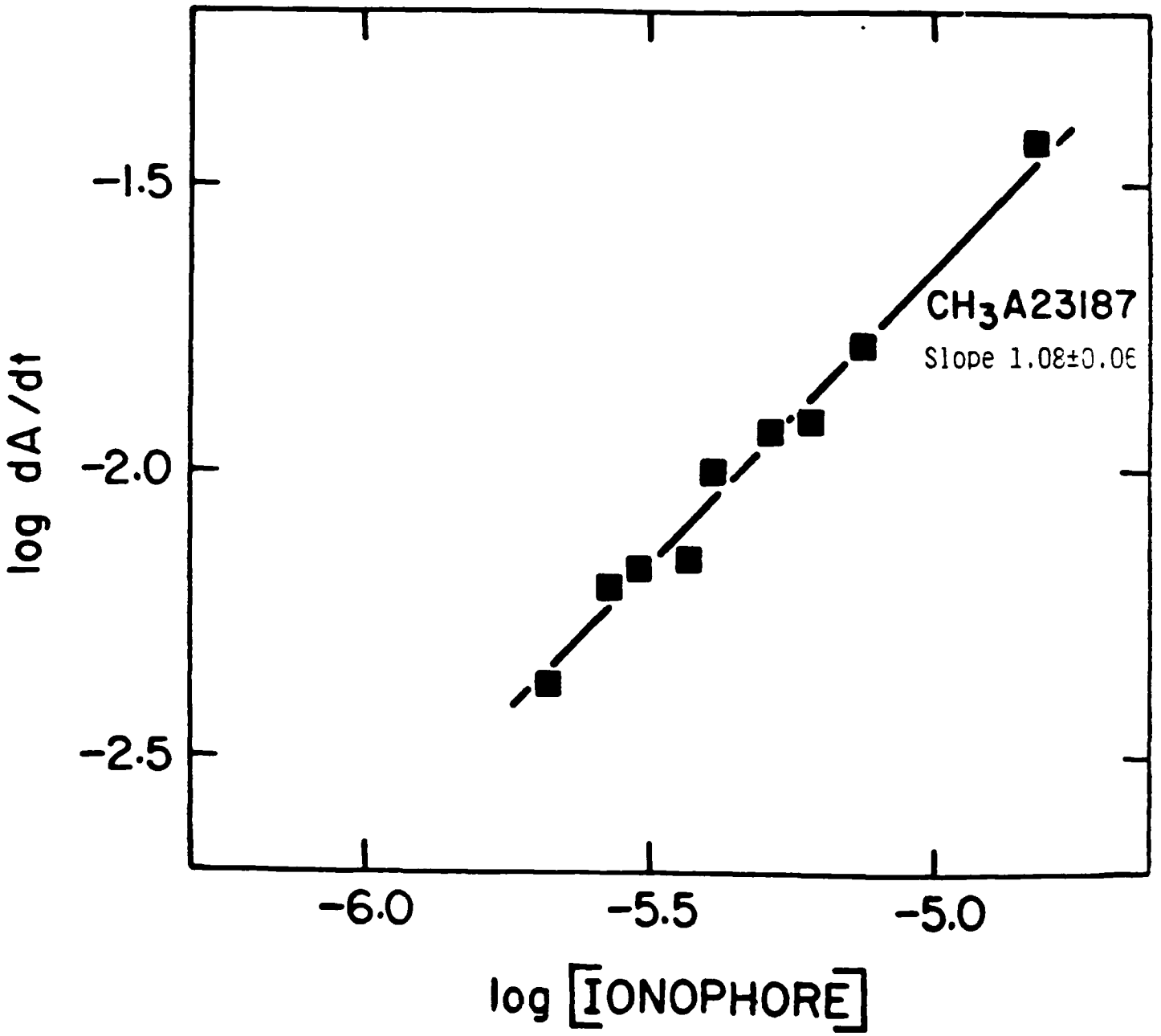


FIGURE 4

The log-log Plot of the Effect of the Ionophore Concentration on the Initial Rates of Ca^{2+} Efflux from LUV

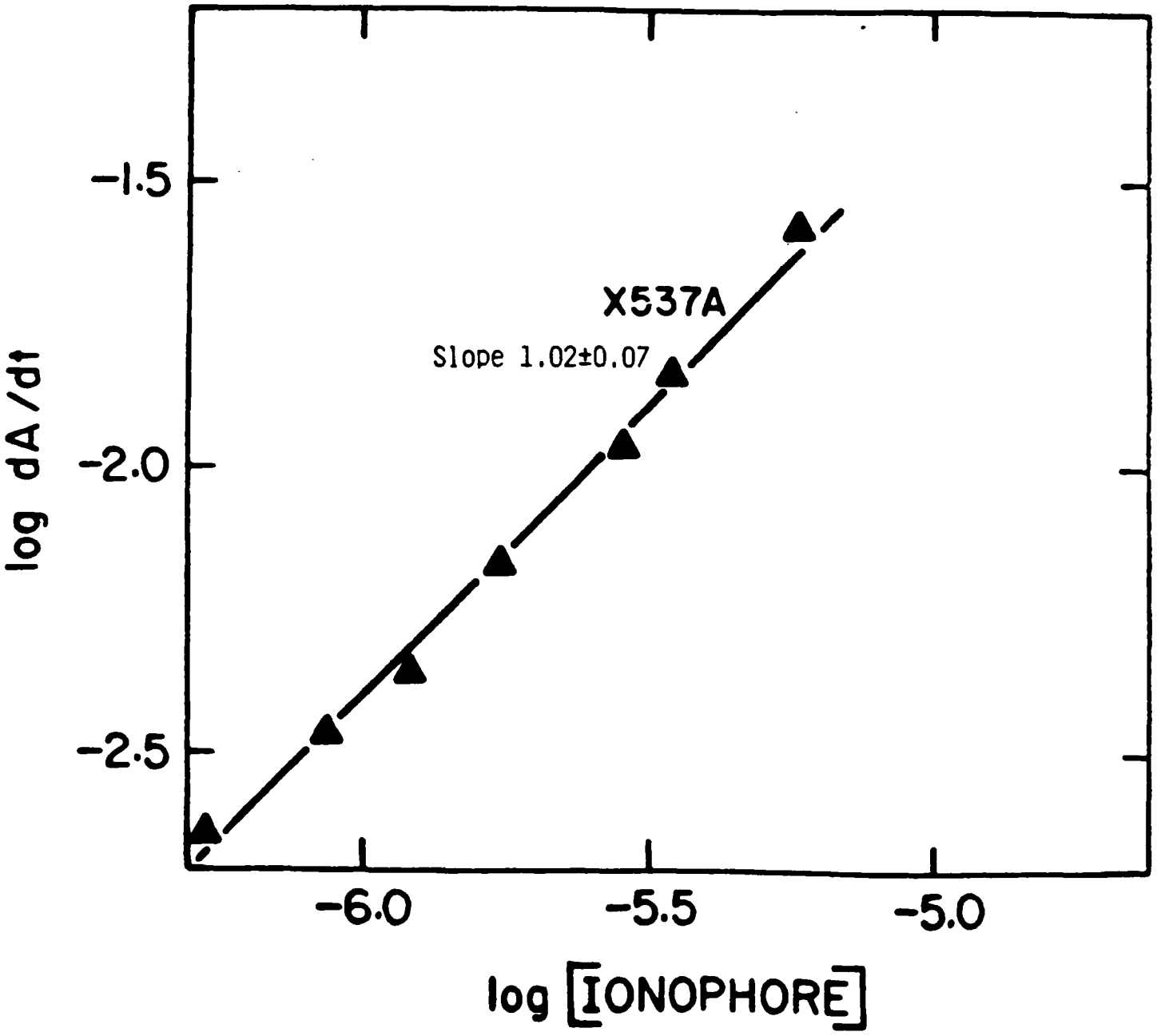


FIGURE 5

Carbon-pivot lariat ethers

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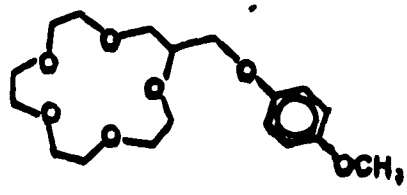

2-((4-methoxyphenoxy)methyl)-15-crown-5

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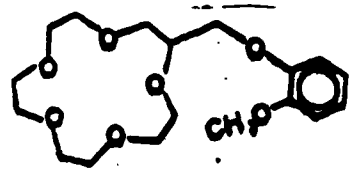

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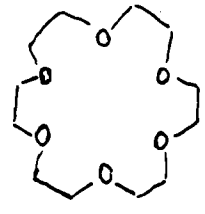

18-crown-6

Nitrogen-pivot lariat ethers

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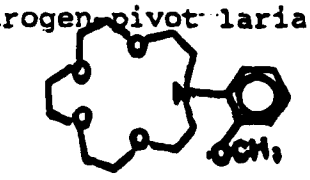

N-(2-methoxyphenyl)monoaza-15-crown-5

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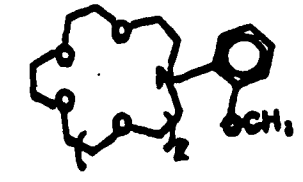

N-(2-methoxyphenyl)monoaza-18-crown-6

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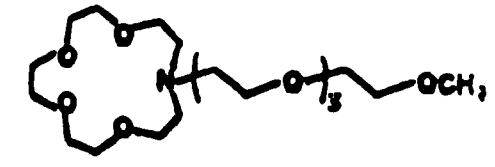

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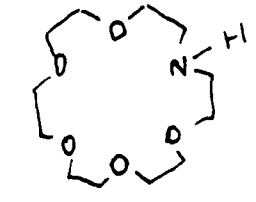
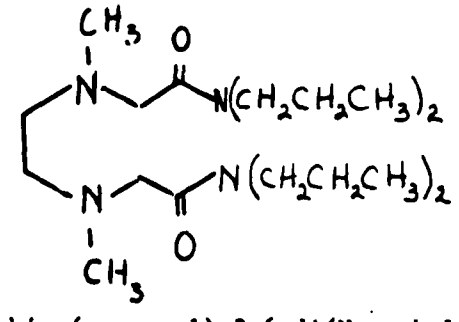


Monoaza-18-crown-6



DMED-PR N,N,N,',N'-Tetrakis-(n-propyl)-3,6-di(N-methyl)azaoctanediamide

FIGURE 6

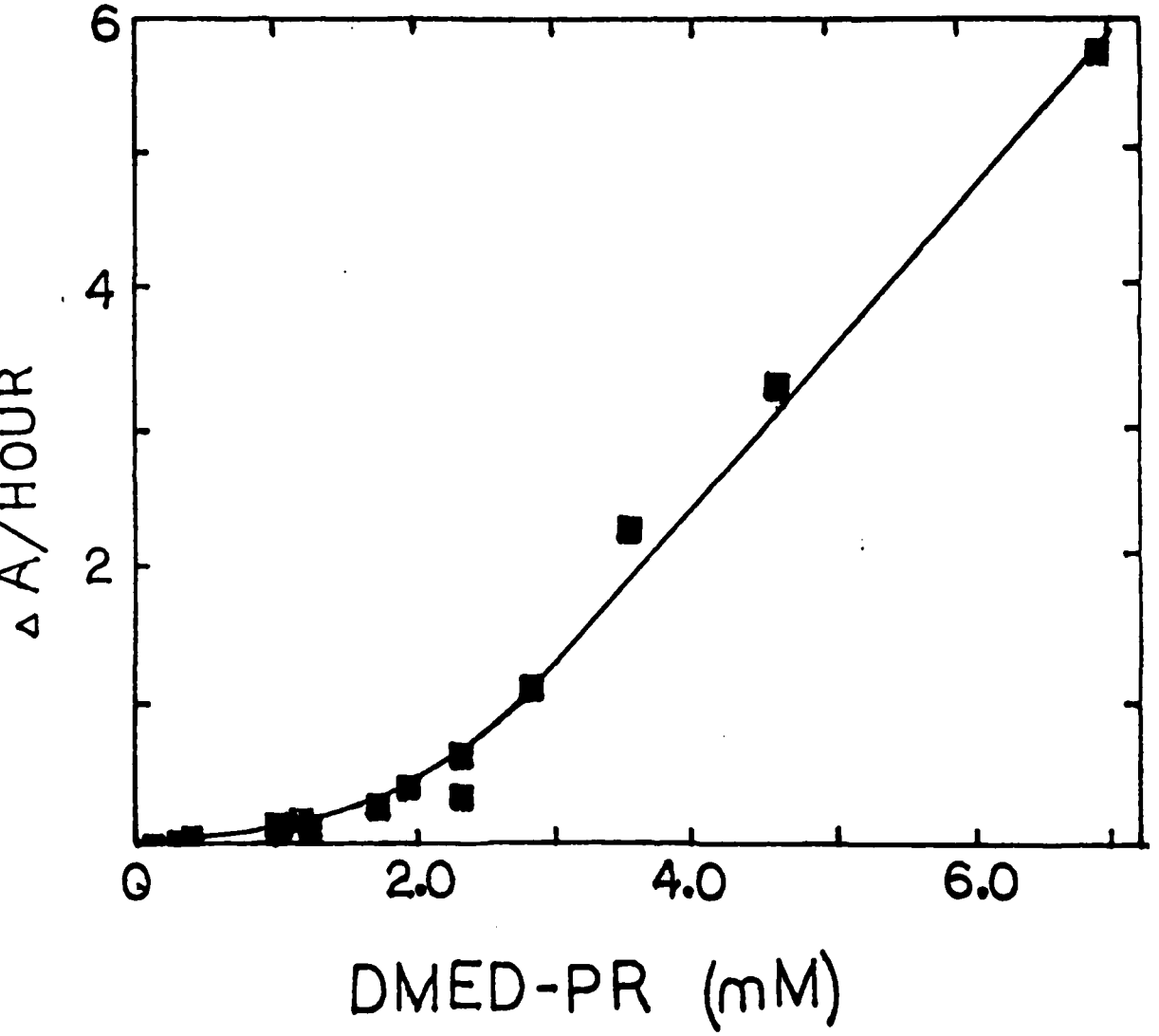
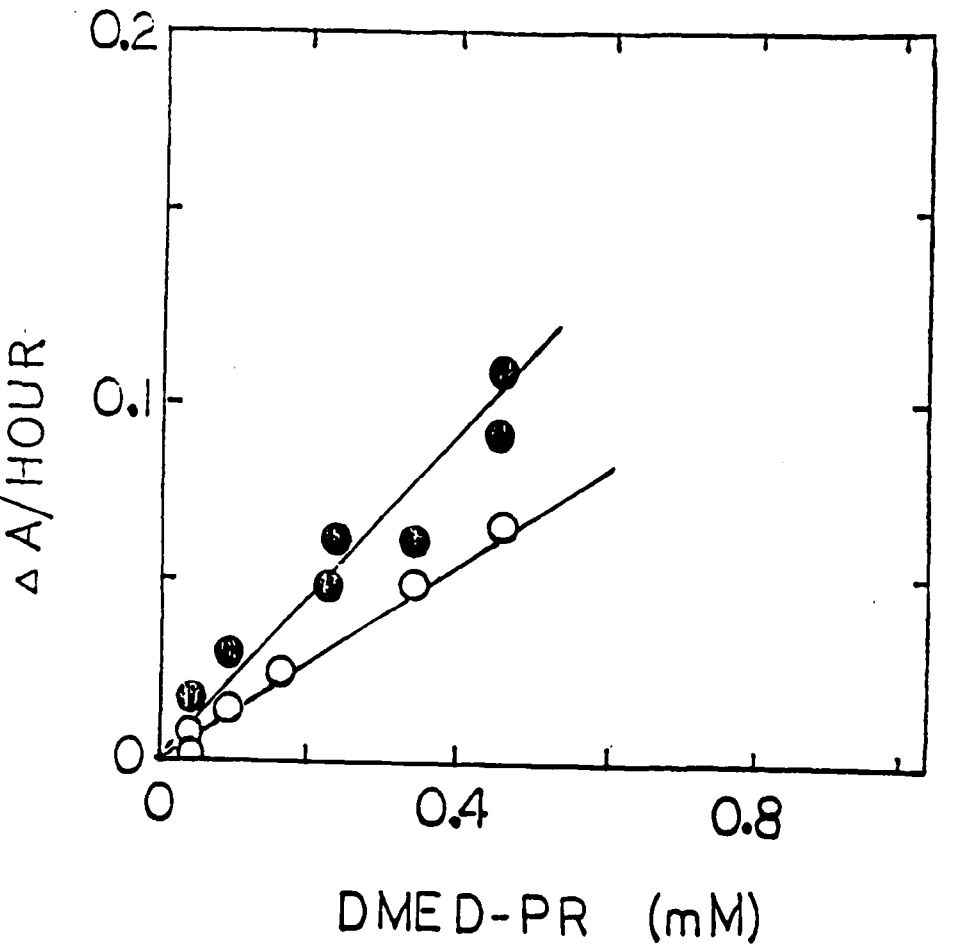


FIGURE 7



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